HILL TOP RESEARCH, INC.

APPENDIX III

COPY OF PROTOCOL

HILL TOP RESEARCH, INC.

PROTOCOL FOR

MODIFIED AOAC GERMICIDAL AND DETERGENT SANITIZING ACTION OF DISINFECTANTS
One Step Cleaner Sanitizer

For: Kimberly-Clark Corporation

HTR Ref.: 01-109643-11

HTR Ref No.: 01-109643-11
Modified AOAC Germicidal and Detergent
Sanitizing Action of Disinfectants Protocol

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1.0 INTRODUCTION

Single-use wipes containing chemical sanitizers suitable for use on lightly soiled, nonporous, food contact surfaces are generally tested by a time kill method where the cidal effect of a specific concentration of chemical agent is measured against both a Gram negative and a Gram positive bacterium over a specified time period. The percent reduction in numbers of test bacteria, containing a 5 % soil load, is calculated as compared to a positive control. Standard practices for testing use the AOAC Germicidal and Detergent Sanitizing Action of Disinfectants Method as described in Chapter 6, Disinfectants, Official Methods of Analysis for AOAC International, 16th Edition, 1995, Section 6.3.03. This method will be modified to test a single-use wipe.

2.0 PURPOSE

To determine the sanitizing action of a wipe containing a chemical agent that can be permitted for use in sanitizing lightly soiled, nonporous surfaces.

3.0 STUDY SPONSOR AND SPONSOR REPRESENTATIVE

Kimberly-Clark Corporation
1400 Holcomb Bridge Rd.
Roswell, GA 30076

Telephone No.: (770) 587-8678
Fax No.: (920) 969-3420

REPRESENTATIVE: Shawn Jenkins

4.0 TEST FACILITY AND INVESTIGATIVE PERSONNEL

Hill Top Research, Inc.
Main and Mill Streets
Miamiville, Ohio 45147

4.0 TEST FACILITY AND INVESTIGATIVE PERSONNEL CON'T.

Telephone No: (513) 831-3114
Fax No.: (513) 831-1217

Study Director:	Kathleen A. Baxter, B.S.
Study Manager:	Jane M. Young, B.S.
Other Investigative Personnel:	Margaret K. Haines, B.S.
	Patricia M. Schario, B.S.
	Elizabeth A. Schumacher, B.S.
Report Writer:	Linda G. Schockman

5.0 APPLICABLE REGULATION

Federal Insecticide, Fungicide and Rodenticide Act (40 CFR Part 158).

6.0 RESEARCH STANDARDS

This study will be run according to Good Laboratory Practice Standards (40 CFR Part 160). An In-Life Phase and Final Report audit will be conducted by the Quality Assurance Unit of Hill Top Research, Inc.

7.0 EXPERIMENTAL DESIGN

Glass baking dishes [pre-sterilized] are inoculated with a specific number of the test bacteria on a 7" X 11" area on the bottom of the dish. A sufficient number of dishes are inoculated with the organism containing a 5% soil load to represent the specified wiped surface area for testing (wiped surface area per dish measures approximately 10.3" X 14" or 1 square foot). The dishes are then wiped with a wipe (12" X 12" or other size as applicable) containing the chemical germicide for a specified period of time. At predetermined exposure time(s), 30 seconds, the remaining chemical agent on the dish is inactivated, and the surviving bacteria are enumerated. The percent reduction in numbers of test bacteria is then calculated. The percent reduction in numbers of bacteria is calculated from a positive control.

8.0 PROPOSED EXPERIMENTAL STARTING AND EXPERIMENTAL TERMINATION DATES

Proposed Experimental Starting Date:	November 26, 2001
Proposed Experimental Termination Date:	November 30, 2001
Proposed Completion Date:	December 28, 2001

9.0 TEST SUBSTANCE IDENTIFICATION

Two lots of the test substance, identified by the sponsor as 7345-76A and 7345-76B and transferred from Hill Top Research Study Nos.: 01-109359-11 and 01-109636-11, will be used for testing. The lots of test substance will be assigned a Hill Top Research code for the generation of the test data.

10.0 TEST SUBSTANCE CHARACTERIZATION

The sponsor will assume responsibility for test substance characterization according to 40 CFR Part 160.105.

11.0 TEST SYSTEM JUSTIFICATION

The test system is designated by federal regulations since it has been used historically for this type of study.

12.0 TEST SYSTEM IDENTIFICATION

The test organism to be used in this study will be *Klebsiella pneumoniae*, ATCC 4352 with 5% Fetal Bovine Serum incorporated as the soil load according to EPA Draft Method Guidance # 02, April 12, 2001. This organism will be assigned a unique code to provide for the correct generation of data.

13.0 TEST PROCEDURE

13.1 The study will be conducted according to the Germicidal and Detergent Sanitizing Action of Disinfectants Method as described in Chapter 6, Disinfectants, Official Methods of Analysis of AOAC International, 16th Edition, 1995 Section 6.3.03 (Appendix I) with modifications. Records

13.0 **TEST PROCEDURE CON'T.**

will be maintained to verify compliance with these procedures, and any approved modifications to these procedures.

13.2 The wipe (test substance) will be tested against *K. pneumoniae*, ATCC 4352 containing a 5% soil load of Fetal Bovine Serum.

13.3 The wipe will be tested as received from the sponsor. **The wipe, 12" X 12," will be removed from roll immediately prior to testing. [Two sheets will be removed from the roll and discarded prior to removing the test sheet.]** One, 12" X 12" wipe will be used to wipe 4 dishes/carriers. One wipe will be used to wipe the specified total test surface area represented by wiping consecutive carriers. The surface area wiped per carrier measures approximately 10.3" X 14" allowing each dish to represent 1 square foot of wiped test surface (4 dishes represent 4 square feet of surface area wiped). **[The wipe will be folded two times in half so that each separate folded portion of the wipe will wipe 1 consecutive dish for the 4 dishes.]**

13.4 Exposure conditions for will be **30 seconds** at 23 ±1°C **after a wiping time of 30 seconds.**

13.5 The neutralizer will be AOAC Neutralizer Blanks with Sea Sand in 400-mL amounts. Neutralizer effectiveness was previously determined under Hill Top Research Study No.: 01-109357-11 with both test organisms using the glass baking dish. The neutralizer will be added to the dish and then the surface will be rubbed [~34 times in the vertical position, ~18 times in the horizontal position, and once around the entire edge (repeat 2 times) in a period of approximately 1 minute] with a sterile rubber policeman to remove the bacteria.

13.6 Other modifications to the AOAC method are as follows:

1) The organism will be harvested using 1.5 mL of AOAC Phosphate Buffer Dilution Water per bottle instead of 3.0 mL as listed in Section 6.3.03D of the AOAC method.

2) A 0.8-mL aliquot of the adjusted test culture suspension (**~1.5 ±0.5 x 10^8**) will be used to inoculate each test surface so that each area of test surface (1 dish/1 square foot) will be inoculated to contain approximately 2.8 X 10^7 CFU/carrier for 1 dish [1 sq. ft.] yielding a count of ~7.5-12.5 X 10^7 CFU/total surface area [4 sq. ft.]. The

13.0 **TEST PROCEDURE CON'T.**

inoculum will be spread evenly over the test piece with the aid of an inoculating loop and then the dish will be allowed to **air dry for 40 min at 37±2 °C and a relative humidity of at least 50%.** The dishes will be loosely covered with aluminum foil during the drying process. Four dishes will be inoculated for each treatment and contact time and four dishes will be inoculated for the for the numbers control.

3) Growth will be confirmed by macroscopic examination rather than the method listed in Section 6.3.03J of the AOAC method.

4) The recovery medium will be Tryptone Glucose Extract Agar with 25 mL/L AOAC Stock Neutralizer. Incubation will be at 35 ± 2 °C for 48 ± 2 hours. Plating will be conducted within thirty minutes of neutralizing the test substance by the Pour Plate Method. Two, 10-mL amounts (10^{-1}) of the AOAC Neutralizer Blanks with Sea Sand will be plated across three plates and duplicate 1-mL and 0.1-mL amounts ($10^{-2}$and 10^{-3} dilutions) will be pour plated. [AOAC Phosphate Buffer Dilution Water with Sea Sand (400-mL) and AOAC Phosphate Buffer Dilution Water (9-mL) and Tryptone Glucose Extract Agar will be used for the numbers controls.] **Plate counts will be conducted in duplicate (a + b) and averaged for each dish. Colony counts per milliliter will be multiplied by 4 to yield Colony forming Units (CFU's) per square foot.**

13.7 **Observations of conditions during the test will be recorded in the study records and the report.**

13.8 Plate counts will be conducted on the expressed fluid from the wipes **immediately** after wiping all 4 of the glass dishes (carriers). Dilutions will be conducted in 9.9 mL or 9.0 mL volumes of AOAC Neutralizer Blanks with plating as outlined in Section 13.6 (4 with modifications to account for use of 100 mL of diluent in place of 400 mL of diluent.

13.0 TEST PROCEDURE CON'T.

13.9 To determine the effectiveness as a cleaner sanitizer, the percent reduction in numbers of test bacteria per square foot of surface area (each dish) will be determined as follows:

For each dish

% Reduction/ 1sq. ft. =

$$\frac{\left[\text{Mean (Avg.) of Numbers Control } \underline{(\text{Dish 1a} + \ldots \text{ Dish 4b})}\right] - \text{Survivors of Individual Dish } \underline{(a+b)}}{\left[\text{Mean (Avg.) of Numbers Control } \underline{(\text{Dish 1a} + \ldots \text{ Dish 4b})}\right]} \times 100$$

13.10 The percent reduction in numbers of test bacteria per total surface area will be determined as follows:

For each of the 4 dish set (4 square feet)

% Reduction/ 4 square feet. =

$$\frac{\left[\text{Sum of Numbers Control } \underline{(\text{Dish 1a} + \ldots \text{ Dish 4b})}\right] - \left[\text{Sum of Survivors } \underline{(\text{Dish 1a} + \ldots 4\text{ b})}\right]}{\left[\text{Sum of Numbers Control } \underline{(\text{Dish 1a} + \ldots \text{ Dish 4b})}\right]} \times 100$$

14.0 STATISTICAL METHOD

No statistical analysis is required to interpret the results of this study.

15.0 REPORT

A draft report will be issued, for review by the sponsor, prior to issuing the final report. The report will include (but not be limited to) identification of the test organism, test procedure, protocol modification (if any), identification of the test material, solvent (if any), test concentration, subculture media, results, and summary.

16.0 DATA RETENTION

The final report and a copy of the raw data will be sent to the sponsor following completion of the study. All records that would be required to reconstruct the study and demonstrate adherence to the Protocol will be maintained. Following completion of the study, the original raw data and the original of the final report will be maintained indefinitely in the form of hard copy to comply with EPA record keeping regulations. The testing

16.0 **DATA RETENTION CON'T.**

facility will retain a copy of these study records in the form of microfilm.

Upon completion of testing, the test substance will be held for one month and then destroyed; or, at your request and cost, sent back to you.

17.0 **NOTICE**

If it becomes necessary to make changes in the approved protocol, the revisions and reasons for change will be documented, reported to the sponsor and will become part of the permanent file for that study.

Similarly, the sponsor will be notified as soon as is practical whenever an event occurs that is unexpected and may have an effect on the validity of the study.

18.0

PROTOCOL APPROVAL FORM
MICROBIOLOGICAL SERVICES DIVISION
HILL TOP RESEARCH, INC.

PROTOCOL TITLE
Modified AOAC Germicidal and Detergent Sanitizing Action of Disinfectants

REFERENCE CODE
DISF\PRO\GERM.SAN\KIMC

PROTOCOL APPROVED FOR: HILL TOP RESEARCH, INC.

BY: Kathleen A. Baxter 11.21.01
Kathleen A. Baxter, B.S. Date
Study Director
Microbiological Services Division

Protocol Approved By (Sponsor):

Signed Date

Signed Date

Kimberly-Clark Corporation 11/26/01

1400 Holcomb Bridge Rd Roswell GA 30076
Address

APPENDIX I

AOAC Method

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Modified AOAC Germicidal and Detergent Sanitizing Action of Disinfectants Protocol

6.3.02

AOAC Official Method 955.17 Fungicidal Activity of Disinfectants

Using *Trichophyton mentagrophytes*

Final Action

(Applicable for use with H_2O-miscible type fungicides used to disinfect inanimate objects.)

A. Test Organism

Use as test fungus typical strain of *Trichophyton mentagrophytes* isolated from dermatophytosis of foot. Strain must sporulate freely on artificial media, presence of abundant conidia being manifested by powdery appearance on surface of 10-day culture, particularly at top of agar slant, and confirmed by microscopic examination. Conidia-bearing mycelium should peel easily from surface of glucose agar. Conidia of required resistance survive 10 min exposure at 20° to phenol dilution of 1–70, but not to one of 1–60. Strain No. 640, ATCC No. 9533, is suitable.

B. Culture Medium

Carry fungus on agar slants of following composition: Glucose 2%, Neopeptone (Difco No. 0119) prepared as a 1% solution, agar 2%, adjusted to pH 6.1–6.3. Use same culture medium to prepare cultures for obtaining conidial suspension, and use fluid medium of same nutrient composition (without agar) to test survival and viability of conidia after exposure to fungicide.

C. Care of Fungus Strain

Store stock culture of fungus on glucose agar slants at 2–5°. At intervals ≤3 months, transfer to fresh agar slants, incubate 10 days at 25–30°, and store at 2–5° until next transfer period. Do not use culture that has been kept at or above room temperature >10 days as source of inoculum for culture. (Cultures may be kept at room temperature to preserve strain and to inoculate cultures if transferred at intervals ≤10 days.)

D. Preparation of Conidial Suspension

Prepare Petri dish cultures by planting inoculum at center of agar plate and incubating culture at 25–30° for ≥10, but ≤15 days. Remove mycelial mats from surface of 5 agar plate cultures, using sterile spatula or heavy flattened wire. Transfer to heat-sterilized glass tissue grinder, 966.04B(e) (*see* 6.3.05), and macerate with 25 mL sterile physiological NaCl solution (0.85% NaCl), or to heat-sterilized Erlenmeyer containing 25 mL sterile saline with glass beads, and shake thoroughly. Filter suspension through sterile absorbent cotton to remove hyphal elements. Estimate density of conidial suspension by counting in hemacytometer and store at 2–10° as stock spore suspension (125–155 × 10^6 conidia/mL) for ≤4 weeks for use in preparing test suspensions of conidia. Standardize test conidial suspensions as needed by diluting stock spore suspension with physiological NaCl solution so that it contains 5 × 10^6 conidia/mL.

E. Operating Technique

Prepare dilutions of fungicide. [Tests are similar to those described in 955.11C (*see* 6.1.01).] Place 5 mL of each fungicide solution and of phenol control solutions in 25 × 150 mm test-culture tubes, arrange in order of ascending dilutions, place tubes in 20° H_2O bath, and let come to temperature. With graduated pipet, place 0.5 mL spore suspension in first tube of fungicidal solution, shake, and immediately replace in H_2O bath; 30 s later add 0.5 mL conidial suspension to second tube. Repeat at 30 s intervals for each fungicidal dilution. If more convenient, run test at 20 s intervals. After 5, 10, and 15 min exposure to fungicide, remove sample from each conidia-fungicide mixture with 4 mm loop and place in 10 mL glucose broth, 955.17B. To eliminate risk of faulty results due to possible fungistatic action, make subtransfers from the initial glucose broth subculture tubes to fresh tubes of glucose broth, using the 4 mm loop before incubation, or make initial subcultures in glucose broth containing either 0.05% sodium thioglycolate, 1.5% isooctylphenoxy-polyethoxy-ethanol, or mixture of 0.07% lecithin (Alcolec Granules, American Lecithin, PO Box 1908, Danbury, CT 06813), and 0.5% polysorbate 80 (Tween 80), whichever gives lowest result. Incubate inoculated tubes at 25–30°. Read final results after 10 days, although indicative reading can be made in 4 days.

Note: Highest dilution that kills spores within 10 min is commonly considered as highest dilution that could be expected to disinfect inanimate surfaces contaminated with pathogenic fungi.

References: Arch. Dermatol. Syphilol. 28, 151(933). J. Bacteriol. 42, 225(1941); 47, 102(1944). JAOAC 37, 616(1954); 38, 274(1955); 56, 308(1973).

6.3.03

AOAC Official Method 960.09 Germicidal and Detergent Sanitizing Action of Disinfectants

Final Action

(Suitable for determining minimum concentration of chemical that can be permitted for use in sanitizing precleaned, nonporous food contact surfaces. Minimum recommended starting concentration is 2–4× this concentration. Test also determines maximum water hardness for claimed concentrations. As control, check accuracy of hard-water tolerance results with pure C_{14} alkyl dimethyl benzyl ammonium chloride at 700 and 900 ppm hardness, and pure C_{16} alkyl dimethyl benzyl ammonium chloride [Cetalkonium Chloride], at 400 and 550 ppm hardness, expressed as $CaCO_3$.)

A. Reagents

(a) *Culture media.*—(*1*) *Nutrient agar A.*—Boil 3 g beef extract, 5 g peptone (from Difco No. 0118 or equivalent; special grades must not be used), and 15 g salt-free agar in 1 L H_2O. Do not use premixed, dehydrated media. Tube, and autoclave 20 min at 121°. Use for daily transfer of test culture. (*2*) *Nutrient agar B.*—Prepare as above but use 30 g agar. Use for growing test cultures in French square bottles. (*3*) *Nutrient agar (AOAC).*—*See* 955.11A(e) (*see* 6.1.01). Use for preparing stock culture slants.

(b) *Subculture media.*—(*1*) Use tryptone glucose extract agar (Difco No. 0002), adding 25 mL stock neutralizer, (c)/L. (*2*) Tryptone glucose extract agar (Difco).

(c) *Neutralizer stock solution.*—Mix 40 g Lecithin (Alcolec Granules, American Lecithin, PO Box 1908, Danbury, CT 06813 [25–50 kg containers only] or Advanced Lecithin Products, PO Box 677, Danbury, CT 06804), 280 mL polysorbate 80, and 1.25 mL phosphate buffer, (e); dilute with H_2O to 1 L and adjust to pH 7.2. Dispense in 100 mL portions and autoclave 20 min at 121°.

(d) *Neutralizer blanks.*—For use with ≤200 ppm quaternary ammonium compound. Mix 100 mL neutralizer stock solution, (c), 25 mL 0.25M phosphate buffer stock solution, (e), and 1675 mL H_2O.

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Table 960.09A Percent Light Transmission at Various Wavelengths Corresponding to Bacterial Concentrations

% Light Transmission with Filters, nm							Average Bacterial Count/mL
370	420	490	530	550	580	650	
7.0	4.0	6.0	6.0	6.0	7.0	8.0	13.0×10^9
8.0	5.0	7.0	7.0	7.0	8.0	9.0	11.5
9.0	6.0	8.0	8.0	8.0	9.0	10.0	10.2
10.0	7.0	9.0	9.0	9.0	11.0	11.0	8.6
11.0	8.0	10.0	10.0	10.0	12.0	13.0	7.7
13.0	9.0	12.0	12.0	12.0	13.0	15.0	6.7

Dispense 9 mL portions into 20 × 150 mm tubes. Autoclave 20 min at 121°.

(e) *Phosphate buffer stock solution.*—0.25M. Dissolve 34.0 g KH_2PO_4 in 500 mL H_2O, adjust to pH 7.2 with 1*N* NaOH, and dilute to 1 L.

(f) *Phosphate buffer dilution water.*—Add 1.25 mL 0.25M phosphate buffer stock solution, (e), to 1 L H_2O and dispense in 99 mL portions. Autoclave 20 min at 121°.

(g) *Test organisms.*—Use *Escherichia coli* ATCC No. 11229 or *Staphylococcus aureus* ATCC 6538. Incubate 24 and 48 h, respectively. Maintain stock cultures on nutrient agar (AOAC), (a)(3), at refrigerator temperature.

B. Resistance to Phenol of Test Cultures

Determine resistance to phenol at least every 3 months by 955.11 (*see* 6.1.01). Resistance of *E. coli* should be equivalent to that specified for *S. typhi* in 955.11D (*see* 6.1.01) and that for *Staph. aureus* equivalent to that specified for this organism in 955.12 (*see* 6.1.02); also, use procedures under 991.48A(b) (*see* 6.2.03) for *Staph. aureus*.

C. Apparatus

(a) *Glassware.*—250 mL wide-mouth Erlenmeyers; 100 mL graduate; Mohr, serological, and/or bacteriological (APHA specification) pipets; 20 × 150 mm test tubes. Sterilize at 180° in hot air oven ≥2 h.

(b) *Petri dishes.*—Sterile.

(c) *French square bottles.*—175 mL, flint glass.

(d) *Water bath.*—Controlled at 25°.

D. Preparation of Culture Suspension

From stock culture inoculate tube of nutrient agar *A*, 960.09A(a)(*1*), and make ≥3 consecutive daily transfers (≤30), incubating transfers 20–24 h at 35–37°. Do not use transfers >30 days. If only 1 daily transfer has been missed, no special procedures are required; if 2 daily transfers are missed, repeat with 3 daily transfers.

Prepare 175 mL French square culture bottles containing 20 mL nutrient agar *B*, 960.09A(a)(2), autoclave 20 min at 121°, and let solidify with bottle in horizontal position. Inoculate culture bottles by washing growth from slant with 5 mL phosphate buffer dilution H_2O, 960.09A(f), into 99 mL phosphate buffer dilution H_2O, and adding 2 mL of this suspension to each culture bottle, tilting back and forth to distribute suspension; then drain excess liquid. Incubate 18–24 h at 35–37°, agar side down. Remove culture from agar surface of 4 or more bottles, using 3 mL phosphate buffer dilution H_2O and glass beads in each bottle to suspend growth. Filter suspension through Whatman No. 2 paper prewet with 1 mL sterile phosphate buffer, and collect in sterile tube. (To hasten filtration, rub paper gently with sterile policeman.) Standardize suspension to give average of 10×10^9 organisms/mL by dilution with sterile phosphate buffer dilution H_2O, 960.09A(f).

If Lumetron colorimeter is used, dilute suspension in sterile Lumetron tube to give % *T* according to Table 960.09A.

If McFarland nephelometer and $BaSO_4$ standards are used, select 7 tubes of same id as that containing test culture suspension. Place 10 mL of each suspension of $BaSO_4$, prepared as indicated in Table 960.09B, in each tube and seal tube. Standardize suspension to correspond to No. 4 standard.

Table 960.09B Preparation of $BaSO_4$ Suspensions Corresponding to Bacterial Concentrations

Standard No.	2% $BaCl_2$ Solution, mL	1% H_2SO_4 (v/v) Solution, mL	Average Bacterial Count/mL
1	4.0	96.0	5.0×10^9
2	5.0	95.0	7.5
3	6.0	94.0	8.5
4	7.0	93.0	10.0
5	8.0	92.0	12.0
6	10.0	90.0	13.5
7	12.0	88.0	15.0

E. Synthetic Hard Water

Prepare *Solution 1* by dissolving 31.74 g $MgCl_2$ (or equivalent of hydrates) and 73.99 g $CaCl_2$ in boiled distilled H_2O and diluting to 1 L. Prepare *Solution 2* by dissolving 56.03 g $NaHCO_3$ in boiled distilled H_2O and diluting to 1 L. *Solution 1* may be heat sterilized; *Solution 2* must be sterilized by filtration. Place required amount *Solution 1* in sterile 1 L flask and add ≥600 mL sterile distilled H_2O; then add 4 mL *Solution 2* and dilute to 1 L with sterile distilled H_2O. Each mL *Solution 1* will give a water equivalent to ca 100 ppm of hardness calculated as $CaCO_3$ by formula:

$$\text{Total hardness as ppm } CaCO_3 = 2.495 \times \text{ppm Ca} + 4.115 \times \text{ppm Mg}$$

pH of all test waters ≤2000 ppm hardness should be 7.6–8.0. Check prepared synthetic waters chemically for hardness at time of tests, using following method or other methods described in 14th ed. of *Standard Methods for the Examination of Water, Sewage, and Industrial Wastes.*

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F. Hardness Method

(a) *EDTA standard solution.*—Dissolve 4.0 g $Na_2H_2EDTA{\cdot}2H_2O$ and 0.10 g $MgCl_2{\cdot}6H_2O$ in 800 mL H_2O and adjust by subsequent dilution so that 1 mL of solution is equivalent to 1 mg $CaCO_3$ when titrated as in (c). Check EDTA solution after preparation or, if commecially purchased, against $CaCO_3$ standard at least every 2 months.

(b) *Calcium standard solution.*—1 mL = 1 mg $CaCO_3$. Weigh 1.00 g $CaCO_3$, dried overnight or longer at 105°, into 500 mL Erlenmeyer and add dilute HCl through funnel until $CaCO_3$ is dissolved. Add 200 mL H_2O, boil to expel CO_2, and cool. Add few drops methyl red indicator and adjust color to intermediate orange with dilute NH_4OH or HCl as required. Transfer quantitatively to 1 L volumetric flask and dilute to volume.

(c) *Determination.*—Dilute 5–25 mL sample (depending on hardness) to 50 mL with H_2O in Erlenmeyer or casserole. Add 1 mL *buffer solution* (67.5 g NH_4Cl and 570 mL NH_4OH diluted to 1 L with H_1O), 1 mL *inhibitor* (5.0 g $Na_2S{\cdot}9H_2O$ or 3.7 g $Na_2S{\cdot}5H_2O$ dissolved in 100 mL H_2O), and 1 or 2 drops *indicator solution* (0.5 g Chrome Black T in 100 mL 60–80% alcohol). Titrate with EDTA standard solution slowly, stirring continuously, until last reddish tinge disappears from solution, adding last few drops at 3–5 s intervals.

Hardness as mg $CaCO_3$/L =

(mL standard solution × 1000)/mL sample

G. Preparation of Samples

Use composition declared or determined as guide to sample weight required for volume sterile H_2O used to prepare 20,000 ppm solution. From this stock dilution, transfer 1 mL into 99 mL of the water to be used in test to give concentration of 200 ppm. In making transfer, fill 1 mL pipet and drain back into stock solution; then refill, to correct for adsorption on glass. After mixing, discard 1 mL to provide 99 mL of the test water in 960.09H.

H. Operating Technique

Measure 99 mL water to be used in test, containing bactericide at concentration to be tested, into chemically clean, sterile, 250 mL wide-mouth Erlenmeyer and place in constant temperature bath until it reaches 25°, or ≥20 min. Prepare duplicate flasks for each germicide to be tested. Also prepare similar flask containing 99 mL sterile phosphate buffer dilution H_2O, 960.09A(f), as "initial numbers" control.

Add 1 mL culture suspension to each test flask as follows: Whirl flask, stopping just before suspension is added, creating enough residual motion of liquid to prevent pooling of suspension at point of contact with test water. Add suspension midway between center and edge of surface with tip of pipet slightly immersed in test solution. Avoid touching pipet to neck or side of flask during addition. Transfer 1 mL portions of this exposed culture to neutralizer blanks exactly 30 and 60 s after addition of suspension. Mix well immediately after transfer.

For "numbers control" transfer, add 1 mL culture suspension to 99 mL sterile phosphate dilution H_2O in same manner. In case of numbers control, plants need be made only immediately after adding and mixing thoroughly ≤30 s. (It is advantageous to use milk pipets to add culture and withdraw samples.)

Plate from neutralizer tube to agar, using subculture medium 960.09A(b)(*1*) for quaternary ammonium compounds and 960.09A(b)(2) with numbers control. Where 0.1 mL portions are planted, use 1 mL pipet graduated in 0.1 mL intervals. For dilutions to give countable plates, use phosphate buffer dilution H_2O, 960.09A(f). For numbers control, use following dilution procedure: Transfer 1 mL exposed culture (1 mL culture suspension transferred to 99 mL phosphate buffer dilution H_2O in H_2O bath) to 99 mL phosphate buffer dilution H_2O, 960.09A(f), (*dilution 1*). Shake thoroughly and transfer 1 mL *dilution 1* to 99 mL phosphate buffer dilution H_2O, 960.09A(f), (*dilution 2*). Shake thoroughly and transfer 1 mL *dilution 2* to 99 mL phosphate buffer dilution H_2O (*dilution 3*). Shake thoroughly and transfer four 1 mL and four 0.1 mL aliquots from *dilution 3* to individual sterile Petri dishes.

For test samples, use following dilution procedure: Transfer 1 mL exposed culture into 9 mL neutralizer, 960.09A(d). Shake and transfer four 1 mL and four 0.1 mL aliquots to individual sterile Petri dishes. For numbers control, use subculture medium 960.09A(b)(2); for tests with quaternary ammonium compounds, use medium 960.09A(b)(*1*). Cool agar to solidify, and then invert and incubate 48 h at 35° before counting.

I. Results

To be considered valid, results must meet standard effectiveness: 99.999% reduction in count of number of organisms within 30 s. Report results according to actual count and % reduction over numbers control. Counts on numbers control for germicide test mixture should fall between 75 and 125 × 10^6/mL for % reductions to be considered valid.

J. Sterility Controls

(a) *Neutralizer.*—Plate 1 mL from previously unopened tube.

(b) *Water.*—Plate 1 mL from each type of water used.

(c) *Sterile distilled water.*—Plate 1 mL.

After counting plates, confirm that surviving organisms are *E. coli* by transfer to brilliant green bile broth fermentation tubes or lactose broth and EMB agar; confirm *Staph. aureus* by microscopic examination.

References: Am. J. Public Health 38, 1405(1948). J. Milk Food Technol. 19, 183(1956). Fed. Regist. 21, 7020(1956). JAOAC 41, 541(1958); 56, 308(1973).

6.3.04

AOAC Official Method 961.02
Germicidal Spray Products as Disinfectants

First Action 1961
Final Action 1964

(Suitable for determining effectiveness of sprays and pressurized spray products as spot disinfectants for contaminated surfaces.)

A. Reagents

Use culture media and reagents specified in 991.47A(a) and (f) (*see* 6.2.02); 991.48A(a) (*see* 6.2.03); and 991.49A(a) and (b) (*see* 6.2.05).

Use as test organisms *Trichophyton mentagrophytes* ATCC No. 9533, prepared as in 955.17D (*see* 6.3.02), to which has been added 0.02 mL octyl-phenoxy-polyethoxy-ethanol (Triton ×100, Union Carbide Corp.)/10 mL suspension to facilitate spreading, *Salmonella choleraesuis* ATCC No. 10708, maintained as in 991.47A(b) (*see* 6.2.02), *Staphylococcus aureus* ATCC No. 6538, maintained as in 991.48A(b) (*see* 6.2.03), and *Pseudomonas aeruginosa* ATCC No.

HILL TOP RESEARCH, INC.
MAIN AND MILL STREETS
MIAMIVILLE, OHIO 45147

PROTOCOL AMENDMENT #1 Modified AOAC Germicidal and Detergent Sanitizing Action of Disinfectants

HTR STUDY NO.: 01-109643-11

SPONSOR & ADDRESS: Kimberly-Clark Corporation
1400 Holcomb Bridge Road
Roswell, GA 30076

SPONSOR'S REPRESENTATIVE: Shawn Jenkins

PROTOCOL AMENDMENT:

1. At the request of the sponsor representative on December 3, 2001, Section 13.7 of the protocol will be amended from "Observations of conditions during the test will be recorded in the study records and the report." to read, "Observations of conditions during the test will be recorded in the study records."

2. At the request of the sponsor representative on November 26, 2001, Section 13.5 of the protocol will be amended from "Neutralizer effectiveness was previously determined under Hill Top Research Study No.: 01-109357-11 with both organisms using the glass baking dish." to read, "Neutralizer effectiveness will be determined with the test organism using the glass baking dish."

APPROVED FOR: HILL TOP RESEARCH, INC.

BY: Kathleen A. Baxter 1.11.02
Kathleen A. Baxter, B.S Date
Study Director
Microbiological Services Division

HILL TOP RESEARCH, INC.
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PROTOCOL DEVIATION #1 Modified AOAC Germicidal and Detergent Sanitizing Action of Disinfectants

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PROTOCOL DEVIATIONS:

1. The numbers controls for *Klebsiella pneumoniae* testing conducted on November 26, 2001 and November 28, 2001 were higher than specified by the protocol. The reduction in numbers of bacteria was greater than 99.9999% in both cases.

2. The numbers of *Klebsiella pneumoniae* for neutralizer testing November 28, 2001 were lower than specified by the protocol (54 vs. 75-125 organisms/mL in the final neutralizing solution).

These deviations did not have an adverse effect on the results of the study, in the opinion of the Study Director.

APPROVED FOR: HILL TOP RESEARCH, INC.

BY: Kathleen A. Baxter 1-11-02
Kathleen A. Baxter, B.S Date
Study Director
Microbiological Services Division